Determination of Steroids in Urine by Micellar HPLC with Detection by Sensitized Terbium Fluorescence

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Efficient Förster energy transfer from steroid donors to a terbium ion acceptor occurs in aqueous solution when the two species are sequestered in sodium dodecyl sulfate micelles. The microorganized medium provides both proximity between donor and acceptor and protection from quenching of terbium fluorescence by water. Detection limits down to 100 pg have been achieved for steroids with an $\alpha,\beta$-unsaturated carbonyl group in the A-ring. This detection strategy has been used in HPLC separations, using either normal- or reversed-phase chromatography with postcolumn addition of detergent and/or terbium, or micellar chromatography with the detergent solution as the mobile phase. This last method proved to be superior, requiring no sample preparation and allowing direct injection of urine onto the HPLC column.

INTRODUCTION

Anabolic steroids, generally used as therapeutic agents in clinical practice, are also widely abused as performance enhancing drugs. Adverse effects on the user, including irreversible changes in the endocrine system, changes in mineral metabolism, alteration of prostatic function, menstrual disorders, and hepatic carcinomas\(^1\)–\(^3\) are well documented. The substances were banned by the International Olympic Committee in 1975, when suitable methods of analysis had been developed. Anabolic steroids also have been administered to cattle and poultry to stimulate meat production. The undesirable side effects ascribed to this practice\(^4\)–\(^6\) have led to strict regulation or total prohibition in most countries. A number of analytical methods are currently in use for steroid testing, including GC, GC/MS, immunoassay methods, and liquid chromatography.\(^7\),\(^8\) Analytical methodologies suitable for the determination of anabolic steroids in different specimen types have to meet two criteria: (1) sensitivity for free steroid down to 1–10 ng/mL in urine and 0.02–1 ng/g in meat samples; (2) possibility of simultaneous determination of different steroids and their metabolites.

Within the framework of analytical methods available today, only the one developed by Donike and Zimmermann\(^9\) meets these requirements. For the determination of androgenic anabolic steroids this involves sample cleanup on a XAD-2 polystyrene resin column, followed by Helix pomatia enzyme treatment for 3 h to liberate the free steroids. These are then extracted into diethyl ether and derivatized with N-methyl-N-(trimethylsilyl)trifluoroacetamide to yield the relatively volatile trimethylsilyl derivates amenable for gas chromatographic separation.\(^4\) The procedure is time consuming and costly, making this method somewhat uneconomical for routine examination of the large numbers of samples that often arise in sporting events.

Among spectroscopic analytical techniques, fluorometry is most suited to achieve the detection limits required in steroid analysis. However, except for a number of aromatic estrogens, the majority of steroids are nonfluorescent. Many steroids react with concentrated sulfuric and phosphoric acid to form fluorescent derivatives,\(^10\) but the lack of selectivity and the formation of a large number of uncharacterized products limits the application of this method in drug testing. Alternatively, a fluorescent tag such as danzylyhydrazine can be attached to the steroid molecule,\(^11\) but this again involves a lengthy derivatization process and can yield extraneous fluorophores, precluding the method from routine analysis. Because of these considerations, the development of a method for steroid determination that gives low limits of detection but requires no derivatization and little or no sample preparation is highly desirable.

It has been known since the 1960s that certain aromatic carbonyls can transfer energy to terbium and europium ions in solution. The luminescence quantum yields of these lanthanides have been shown\(^12\) to be substantially increased when a highly absorbing sensitizer is used. Recent work has shown the analytical utility of lanthanide sensitization in rotoreta analysis.\(^13\) The energy-transfer efficiency depends on the proximity of the excited electronic level of the donor and, for instance, the d level of terbium. For the trivalent terbium ion to act as a good acceptor, the following conditions must be satisfied: (1) the excited energy level of the donor should be slightly above the 6d level of terbium; (2) a nonaqueous environment should be maintained to prevent the strong quenching of terbium fluorescence by water; and (3) in the case of Förster-type energy transfer, the donor and the acceptor should be less than ~100 Å apart.

The structures of some important steroids are shown in Figure 1, all containing an $\alpha,\beta$-unsaturated carbonyl group in the A-ring. This structural feature is common to many anabolic steroids of abuse, since they are generally testosterone and cortisone, and analytical methods must therefore provide selectivity for these species.

\(^{(6)}\) McMartin, K. E. Environ. Pathol. Toxicol. 1978, 1, 279.
\(^{(7)}\) Marx, J. J. Science 1978, 202, 1270.
The first excited singlet level of a typical semiquinonoid steroid \(^{14}\) is at 41 666 cm\(^{-1}\), corresponding to strong absorption at 240 nm, with a molar absorptivity of \(\sim 18\ 000\ \text{L mol}^{-1}\ \text{cm}^{-1}\). The important feature for lanthanide sensitization is the existence of a triplet level at 26 041 cm\(^{-1}\), which produces steroid phosphorescence that can be observed at 77 K. \(^{14}\) At room temperature this triplet energy level can be used to efficiently pump the \(5\delta_3\) level of the terbium ion, which occurs at 26 000 cm\(^{-1}\). This, after relaxation to \(\delta_4\), undergoes a radiative transition to the \(\gamma\) ground level, resulting in the characteristic terbium ion fluorescence. We have found that the excitation of terbium by means of energy transfer from steroids results in 183-fold fluorescence enhancement. Kallistratos et al. \(^{15}\) have also observed increased terbium fluorescence in the presence of steroids with a semiquinonoid structure. However, their investigation was limited to ethanolic solutions and did not address the fluorescence-quenching problems in body fluids. The lowest detected quantity of steroid reported was 0.5 ng/mL, and no attempts were made to impart specificity to the method.

We have effectively overcome the problem of terbium fluorescence quenching in aqueous solution (such as urine) by compartmentalizing the terbium acceptor and the steroid donor in sodium dodecyl sulfate micelles. Mwalupindi et al. \(^{16}\) have recently investigated a comparable system comprising lanthanide ions encapsulated with reverse micelles. In the present aqueous system, the micelles not only exclude water, but also permit efficient energy transfer because of enforced proximity of the donor and acceptor. It has enabled us to achieve detection limits in the 0.5 ng/mL range for the semiquinonoid steroids studied. By utilizing this mode of detection with high-performance liquid chromatography, we have developed a sensitive method of steroid analysis in urine at the ppb level. The use of a micellar HPLC mobile phase further simplified the procedure by permitting the direct injection of urine into the column, without sample preparation. Mechanistic studies suggest a Förster-type energy transfer from the steroid to terbium in micellar media.

**EXPERIMENTAL SECTION**

**Chemicals.** Testosterone, methyltestosterone, bolasterone, testosterone acetate, cortisone, and progesterone were purchased from Sigma (St. Louis, MO), and Tb(NO\(_3\))\(_3\)·5H\(_2\)O and Eu(NO\(_3\))\(_3\)·6H\(_2\)O (both 99.99%) were obtained from Aldrich (Milwaukee, WI). All were used without further purification. Ultrapure sodium dodecyl sulfate (SDS; 99.99%) was purchased from J. T. Baker (Phillipsburg, N.J) and was used as received. All organic solvents used were of HPLC grade and were obtained from Aldrich. Pyrene used for mechanistic studies was purchased from Sigma, recrystallized twice from a absolute ethanol, and then purified by cold finger sublimation. Deionized water treated with a 0.22-μm Millipore filter system to 18 MΩ cm resistivity was used throughout.

**Instrumentation.** Fluorescence spectra were recorded on a Perkin-Elmer MFP-66 fluorescence spectrophotometer, which provides corrected excitation and emission spectra. The instrument also served as a fluorescence detector for HPLC separations. A Varian Model 5000 HPLC, equipped with a Waters variable-volume injector (20 μL to 2 mL) was used for the chromatographic work. This instrument provides column temperature control with ± 0.1°C. A Waters Model 510 HPLC pump was used for the postcolumn addition of Tb(III) solution into the eluent stream. The complete mixing of two solutions was ensured by passing them through a tightly coiled 50-cm capillary tube. A 0.2-mL, 1-cm path length semimicro quartz flow cell was used to measure HPLC eluent fluorescence.

**Sample Handling.** Prior to reversed and normal-phase HPLC separations, samples were cleaned and preconcentrated by solid-phase extraction on a 1-mL Supelclean LC-18 SPE tube. The method, suggested by Supelco Inc., \(^{17}\) effected a solid-phase extraction of anabolic steroids from urine. Typically a 10-mL urine sample spiked with each steroid to give a concentration of 1 ppb was passed through the SPE tube at a constant flow rate of 2 mL/min. The packing was then successively washed with 4 mL of 0.025 M pH 8 sodium borate buffer, 4 mL of 40% methanol, and 4 mL of 0% acetone. Finally the steroids were eluted with two 500-μL aliquots of 73% methanol. A uniform flow rate was achieved by the application of a vacuum, which also ensured the complete elution of the solvent. For reversed-phase and micellar chromatography, 200 μL of the above solution was directly injected onto the column, whereas for normal-phase chromatography the methanol solvent was evaporated under nitrogen at 40 °C and the residue reconstituted with 1.0 mL of mobile phase. For micellar liquid chromatography, 200 μL of urine was directly injected onto the column.

**Chromatography.** (a) Reversed Phase. The separations were carried out on a 5-μm, 25-cm C-18 column (4.6-mm i.d.)


supplied by Rainin, using 1:1 CH₂CN-H₂O as the eluent. Isocratic elution was carried out at 25 °C. A 0.03 M Tb(NO₃)₃ solution in 0.1 M SDS was introduced into the eluent stream as it emerged from the column.

(b) Normal Phase. A 5-μm, 25-cm silica Microsorb column (4.5-mm i.d.) supplied by Rainin was used. The separation was carried out by isocratic elution with 6:4 ethyl acetate-cyclohexane. A 0.03 M Tb(NO₃)₃ solution in ethyl acetate was introduced into the eluent stream as it emerged from the column.

(c) Micellar Chromatography. Micellar chromatography was carried out on the C-18 column described above. The aqueous mobile phase contained 0.01 M Tb(NO₃)₃, 0.1 M SDS, and 20% acetonitrile. The acetonitrile was added as the organic modifier to enhance mass transfer of the solute from the stationary phase to the mobile phase. Column temperature was maintained at 40 °C during elution.

Standard solutions (10 μg/mL) of each steroid were prepared by dissolving the appropriate amount in 10 mL of absolute methanol. The standard solutions were stored at 4 °C in amber glass flasks.

RESULTS AND DISCUSSION

Addition of SDS to a Tb(NO₃)₃ solution results in the initial precipitation of terbium dodecyl sulfate (T(DS)₉), which redissolves on addition of further SDS. Earlier reports indicated no change in terbium ion fluorescence intensity as a function of SDS concentration and led workers to conclude that Tb³⁺ retains water in its coordination sites and is predominantly solubilized in the highly charged Stern layer of the micelle. However, we have found a modest (60%) increase in terbium ion fluorescence at the critical micelle concentration (cmc) of SDS, which corresponds to the typical increase in lanthanide quantum yield when passing from water to an organic medium. This suggests that the neutral T(DS)₉ is solubilized in the micelle, probably by accommodation in the micellar palisade layer. The ion experiences shielding from the aqueous medium and is brought into enforced proximity to the donor that is also micellized.

The molar absorbivities of the various steroids and Tb³⁺ in 0.1 M SDS are listed in Table I. At 245 nm, the absorbance of the steroid is approximately 7000-fold greater than that of terbium. Excitation of a solution containing 0.01 M Tb(NO₃)₃ dissolved in 0.1 M SDS at 245 nm results in weak terbium ion fluorescence comprising bands at 495, 548, 594, and 624 nm. These peaks are attributed to the 5d₄ transitions, respectively. Addition of 1 μg/mL testosterone to this solution resulted in a 120-fold enhancement of the emission intensity (Figure 2). Steroids such as androsterone that lack the α,β-unsaturated carbonyl chromophore, do not produce increased terbium fluorescence. Enhancement was also observed in Eu³⁺, Sm³⁺, and Dy³⁺ fluorescence, but the effect was strongest with terbium, due to the close energy match between the donor triplet level and the terbium excited singlet state (Figure 3). Among the different anabolic steroids studied, bolasterone and testosterone acetate produced the greatest enhancement.

Mechanism. Donor–acceptor proximity is essential for efficient energy transfer, and in many cases, this is ensured by complex formation between the two species. However, recently Tran and Zhang reported Förster energy transfer from the benzoate ion to four lanthanides, leading to a 60-fold enhancement in fluorescence. We have concluded that energy transfer from semiquinonoid steroids to lanthanides in micellar solutions is also the Förster type, in view of the following considerations.

(a) Proton NMR. The vinylic proton at C₄ in the A-ring of testosterone resonates at 5.5 ppm in CD₃CN. Complex formation with terbium at this location is expected to result in a sizable downfield shift (lanthanide shift). However, the spectrum of a solution containing 0.003 M testosterone, 0.013 M Tb³⁺, and 0.13 M SDS in CD₃CN-D₂O (2:8) showed no shift whatever in the resonance of this proton.

(b) Fluorescence. The terbium ion fluorescence at 547 nm was monitored as a function of SDS concentration in 20% acetonitrile–water, with the testosterone and terbium concentrations kept constant at 2.5 μg/mL and 0.001 M, respectively. At these concentrations in this solvent, T(DS)₉ remained soluble at all SDS concentrations. A plot of terbium ion emission intensity vs SDS concentration (Figure 4) shows a positive slope until the cmc was reached. (The cmc was at 0.05 M in this solvent, which was confirmed by independent means—vide infra.) At concentrations above the cmc, the fluorescence intensity sharply decreased. This behavior is not consistent with complex formation, but rather suggests a Förster transfer scenario. At concentrations below the cmc, no detergent micelles are present, and quenching of terbium fluorescence by water leads to weak emission. As the cmc is

Table I. Molar Absorptivities of Steroid Donors and Terbium Acceptor at 245 nm in 0.1 M SDS Solution

<table>
<thead>
<tr>
<th>steroid</th>
<th>molar absorp</th>
<th>molar absorp</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(L mol⁻¹ cm⁻¹)</td>
<td>(L mol⁻¹ cm⁻¹)</td>
</tr>
<tr>
<td>testosterone</td>
<td>14 723</td>
<td>13 364</td>
</tr>
<tr>
<td>methyltestosterone</td>
<td>13 870</td>
<td>14 342</td>
</tr>
<tr>
<td>testosterone acetate</td>
<td>14 359</td>
<td>2</td>
</tr>
</tbody>
</table>

approached, preemilic aggregation\textsuperscript{22} affords an increasing measure of protection for terbium against quenching, leading to an increase in emission intensity. At the cmc, both T(DS)\textsubscript{3} and testosterone are localized in the micellar interior, shielded from quenching and at the same time in close proximity to each other. This results in a sharp increase in fluorescence intensity. At detergent concentrations above the cmc, both the concentration and the size of the micelles increase, decreasing the probability of finding a donor and an acceptor in the same micelle and increasing the distance between them where this is the case. The net effect is a rapidly decreasing energy-transfer efficiency and sensitized fluorescence intensity. The observed behavior therefore suggests Förster energy transfer.

The photophysical mechanism can be envisaged as the radiative excitation of the steroid to the first excited singlet state, followed by the intersystem crossing to the first triplet state, followed by the intersystem crossing to the first triplet state, followed by the intersystem crossing to the first triplet state.

Energy enhancement (EN) and energy-transfer efficiency\textsuperscript{23} (ET) in the micellar system was determined by

$$EN = \frac{F_{\text{pre}}}{F_{\text{abs}}}$$

$$ET = \frac{A_{\text{occ}}}{A_{\text{don}}} (EN - 1)$$

where $F_{\text{pre}}$ is the fluorescence intensity in the presence of steroid and $F_{\text{abs}}$ is the fluorescence intensity measured in the absence of steroid. $A_{\text{occ}}$ and $A_{\text{don}}$ are the absorbances of acceptor and donor, respectively (at 245 nm in the present case). The calculated values for EN and ET are listed in Table II. They correlate well with the detection limit of the steroids that are also shown in the table. The low values obtained for testosterone are probably due to the relatively polar nature of this steroid, which leads to a lower concentration in the micelles.

In organic solvents energy transfer appears to occur via prior complexation of Tb\textsuperscript{3+} with the $\alpha,\beta$-unsaturated carboxyl group of the steroid. This is supported by the observation that testosterone absorption undergoes a 20-nm bathochromic shift upon complexation with Tb\textsuperscript{3+} in acetonitrile. Pure testosterone absorbs at 240 nm, but this shifts to 260 nm when Tb\textsuperscript{3+} is added (1:3). Furthermore, the NMR spectrum of testosterone in the presence of terbium (1:10) shows the complete disappearance of the vinyllic C\textsubscript{4} proton resonance. This indicates that terbium is complexed at the steroid A-ring, resulting in an exiplex that undergoes internal energy transfer to the coordinated metal ion. The complex formed is light sensitive: a 50\% decrease in fluorescence intensity was observed when the solution was irradiated at 260 nm for 1 h.

The above discussion leaves unanswered the question of why Tb\textsuperscript{3+} and semiquinonoid steroids form complexes in organic media but not in micelles, which are comparable environments. The more structured interior of the micelle is probably the cause of this: solubilized T(DS)\textsubscript{3} is likely to align itself with the palisade layer, with the metal end near the solution boundary. Total exposure of terbium to the aqueous phase is not indicated, since there is no evidence of water quenching. However, while free mutual accessibility is not available for complexation in the structured environment, the diffuse close encounters necessary for Förster transfer are easily achieved.

**Chromatographic Separation.** (a) Normal and Reversed Phase. Separation of various steroids by both types of HPLC has been demonstrated.\textsuperscript{24,25} We have used both with postcolumn addition of Tb\textsuperscript{3+} (normal and reversed phase) and detergent (reversed phase only), as indicated under Experimental Section. The 0.03 M Tb\textsuperscript{3+} concentration constitutes a modest excess of the ion relative to the micelle concentration, which lies around 0.0016 M in a 0.10 M SDS solution. It produces the optimum fluorescence signal under the experimental conditions. Baseline separation was achieved with these techniques (Figure 5), and analytical figures of merit are listed in Table III. The detection limits are comparable to those obtained with GC/MS. Both types of chromatography require prior analyte extraction from the sample matrix, which is usually urine. In normal-phase HPLC it is necessary because of sample incompatibility with the mobile phase, while in reversed-phase HPLC precipitation of urinary proteins in the column leads to blockage and makes direct injection impossible. Solid-phase extraction on an Amberlite XAD-2 resin has been used for this purpose in the past,\textsuperscript{26} but in the present case, the method gave only 60–70\% steroid recovery. The process was found to be time consuming, taking as long as 3 h. Solvent extraction with ethyl acetate proved equally unsatisfactory since it produced slow-settling dispersions with urine. Preconcentration with C-18 solid-phase extraction cartridges or columns has been shown to be fast and highly efficient,\textsuperscript{17} giving recoveries in the 95–98\% range. It separates the anabolic steroids from impurities in three ways: (i) selective extraction—the initial conditions are selected such that the steroids are retained, while polar impurities such as uric acid pass unimpeded; (ii) selective

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
steroid & EN factor & % energy transfer & detection limit (pg/mL) \\
\hline
testosterone & 124 & 48.6 & 300 \\

methyltestosterone & 114 & 49.3 & 400 \\
cortisone & 33 & 16.1 & 3000 \\

bolasterone & 183 & 86.2 & 100 \\
testosterone acetate & 183 & 86.2 & 100 \\
progesterone & 158 & 69.0 & 200 \\
\hline
\end{tabular}
\end{table}
versed-phase HPLC separations with terbium fluorescence superiority of C-18.

by the two extraction procedures, clearly demonstrating the multiple applications. Table IV list the recoveries obtained is the loss in the extraction efficiency of the cartridges in the very small volume of solvent required with the former to elution—the retained steroids are selectively eluted with methanol. The main advantage of octadecyl-coated silica elute the analytes, which prevents dilution. A disadvantage problem can be circumvented by the use of micellar chro-

but still require fairly extensive sample preparation. This

matography. The unique ability of detergent micelles to

dissolve urinary proteins has been exploited to develop novel methods of analysis for biologically important compounds in body fluids. Recently Berthod et al. demonstrated the application of micellar liquid chromatography to the analysis of drugs of abuse in urine. The use of UV detection, however, limited the sensitivity for the two steroids studied by these authors to the ppm level. The observed energy transfer from steroids to lanthanides in SDS solutions prompted us to explore the possibility of using the micellar medium as the chromatographic mobile phase. The routinely used organic modifier n-propanol resulted in poor resolution of the steroids, as did other alcohols (C1-C5) added in organic modifier n-propanol resulted in poor resolution of the steroids, as did other alcohols (C1-C5) added in quantities ranging from 6% to 15% to the mobile phase. Satisfactory separation was obtained with a mobile phase containing 20% acetonitrile as the organic modifier. A typical chromatogram showing the separation of testosterone, methyltestosterone, bolasterone, progesterone, and testosterone acetate is shown in Figure 6. For this, urine was spiked with the steroids to give final concentrations of 100–300 ppb for each, and the sample was directly injected onto the column. This clearly simplifies and speeds up the procedure, while

Table III. Analytical Results of HPLC Steroid Separations

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Det limit (pg/inj)</th>
<th>Linear range (ng)</th>
<th>Corr coeff</th>
<th>Det limit (ng/inj)</th>
<th>Linear range (ng)</th>
<th>Corr coeff</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>170</td>
<td>1.7-1000</td>
<td>0.999</td>
<td>1.0</td>
<td>1-500</td>
<td>0.999</td>
</tr>
<tr>
<td>Methyltestosterone</td>
<td>200</td>
<td>1.0-1000</td>
<td>0.997</td>
<td>1.0</td>
<td>1-500</td>
<td>0.991</td>
</tr>
<tr>
<td>Testosterone acetate</td>
<td>260</td>
<td>1.0-1000</td>
<td>0.967</td>
<td>0.8</td>
<td>1-600</td>
<td>0.996</td>
</tr>
<tr>
<td>Bolasterone</td>
<td>200</td>
<td>2.0-1000</td>
<td>0.998</td>
<td>0.8</td>
<td>1-600</td>
<td>0.982</td>
</tr>
<tr>
<td>Progesterone</td>
<td>160</td>
<td>1.6-1000</td>
<td>0.950</td>
<td>1.0</td>
<td>1-500</td>
<td>0.998</td>
</tr>
</tbody>
</table>

Table IV. Extraction Efficiency of Steroids from Urine on Solid-State Extraction Cartridges

<table>
<thead>
<tr>
<th>Steroid</th>
<th>% Extraction polystyrene</th>
<th>XAD-2 resin</th>
<th>C-18 bonded silica</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>62.3 ± 3.2</td>
<td>75.0 ± 3.0</td>
<td></td>
</tr>
<tr>
<td>Methyltestosterone</td>
<td>61.4 ± 4.0</td>
<td>80.0 ± 4.0</td>
<td></td>
</tr>
<tr>
<td>Testosterone acetate</td>
<td>59.1 ± 5.0</td>
<td>77.0 ± 2.5</td>
<td></td>
</tr>
<tr>
<td>Bolasterone</td>
<td>65.7 ± 2.8</td>
<td>81.0 ± 3.0</td>
<td></td>
</tr>
<tr>
<td>Progesterone</td>
<td>67.3 ± 3.0</td>
<td>78.0 ± 2.0</td>
<td></td>
</tr>
</tbody>
</table>

Figure 5. HPLC separation of steroids with detection by postcolumn terbium addition: (A) normal phase; (B) reversed phase. Added 0.03 M Tb(III) solution in 0.1 M SDS at 0.5 mL/min. Steroids injected: (a) testosterone acetate (1 ng); (b) progesterone (1 ng); (c) bolasterone (0.5 ng); (d) methyltestosterone (1 ng); (e) testosterone (1 ng).

washing—the less polar impurities such as bilirubin are selectively removed by washing the column sequentially with sodium borate buffer, methanol, and acetone; (iii) selective elution—the retained steroids are selectively eluted with methanol. The main advantage of octadecyl-coated silica over polystyrene XAD-2 resin for solid-phase extraction is the very small volume of solvent required with the former to elute the analytes, which prevents dilution. A disadvantage is the loss in the extraction efficiency of the cartridges in multiple applications. Table IV list the recoveries obtained by the two extraction procedures, clearly demonstrating the superiority of C-18.

(b) Micellar Chromatography. The normal- and reversed-phase HPLC separations with terbium fluorescence detection discussed above give good sensitivity and selectivity, but still require fairly extensive sample preparation. This problem can be circumvented by the use of micellar chromatography. The unique ability of detergent micelles to

Figure 6. Steroid separation by micellar liquid chromatography: (A) standard solution (20 ng of each steroid); (B) 200-μL urine sample spiked with 300 ng/mL (a) testosterone and (b) methyltestosterone and 100 ng/mL (c) bolasterone, (d) progesterone, and (e) testosterone acetate.

Table V. Analytical Results Obtained with Steroid Separation Directly in Urine by Micellar Chromatography

<table>
<thead>
<tr>
<th>steroid</th>
<th>detection limit (ng/200-μL injection)</th>
<th>% rec (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>testosterone</td>
<td>10</td>
<td>90 ± 4.0</td>
</tr>
<tr>
<td>methyltestosterone</td>
<td>10</td>
<td>95 ± 3.3</td>
</tr>
<tr>
<td>testosterone acetate</td>
<td>2</td>
<td>96 ± 2.8</td>
</tr>
<tr>
<td>bolasterone</td>
<td>2</td>
<td>92 ± 3.0</td>
</tr>
<tr>
<td>progesterone</td>
<td>2</td>
<td>95 ± 4.2</td>
</tr>
</tbody>
</table>

retaining good sensitivity and selectivity. A detection limit of 0.5 ppb was easily achieved with standard solutions of the steroids studied. Linear ranges extended over 2 orders of magnitude, and correlation coefficients were above 0.99. We were able to detect the steroid at levels as low as 20 pg/injection, but a somewhat unstable baseline due to source fluctuations gave unsatisfactory reproducibility with these extremely small amounts. The practical detection limits in urine are listed in Table V. These results were achieved entirely without sample preparation, and detectability can be further reduced below 1 ppb by carrying out prior sample cleanup with solid-phase extraction.

The existence of detergent micelles at the relatively high concentration of organic modifier used was confirmed by measuring the \( I_1/I_2 \) vibronic band ratio of pyrene as a function of SDS concentration. This ratio is sensitive to the immediate environment of the fluorescent probe and is well established as an indicator of micellization. The 20% acetonitrile eluent was spiked with 5 \( \times 10^{-4} \) M pyrene, and its 337-nm emission was measured with SDS concentrations in the range 1.0 \( \times 10^{-4} \)–0.3 M. The plot of \( I_1/I_2 \) vs [SDS] showed a break at [SDS] = 0.05 M, indicating that the cmc had been reached (Figure 7). It should be noted that the value in the mixed solvent is somewhat higher than the corresponding one (0.008 M) in water.

CONCLUSIONS

The sensitivity afforded by sensitized lanthanide fluorescence in micellar media has been coupled with the selectivity of HPLC to provide a viable alternative to GC/MS for steroid analysis in body fluids. The advantage of the method lies in its speed and simplicity, while detection limits are retained in the low ppb range. Calibration curve linearity extends over 2 orders of magnitude with correlation coefficients around 0.99. Analysis of urine has shown that the method does not suffer from serious interferences. The micellar medium can be introduced into the system in two ways: in reversed-phase HPLC by postcolumn addition and in micellar HPLC directly as the mobile phase. The former method gives somewhat better measurement sensitivity, but it requires prior extraction of the steroids and the instrumentation for postcolumn addition involves a further degree of complication. Micellar HPLC with terbium fluorescence detection should therefore be regarded as the superior analytical method for semiquinonoid steroids, providing sensitivity, selectivity, simplicity, and low cost. Application of this method to steroids not possessing the \( \alpha, \beta \)-unsaturated carbonyl moiety, such as stanozolol, is presently under investigation. We have also found that amino acids such as lysine, glycine, tyrosine, and histidine show energy transfer to Tb\(^{3+} \) and are in the process of developing the method for these compounds.

Figure 7. Variation of pyrene \( I_1/I_2 \) ratio with SDS concentration in 20% acetonitrile–water.

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